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Genetic diversity and antifungal activity of native *Pseudomonas* isolated from maize plants grown in a central region of Argentina

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ABSTRACT

Pseudomonas strains producing antimicrobial secondary metabolites play an important role in the biocontrol of phytopathogenic fungi. In this study, native *Pseudomonas* spp. isolates were obtained from the rhizosphere, endorhizosphere and bulk soil of maize fields in Córdoba (Argentina) during both the vegetative and reproductive stages of plant growth. However, the diversity based on repetitive-element PCR (rep-PCR) and amplified ribosomal DNA restriction analysis (ARDRA) fingerprinting was not associated with the stage of plant growth.

Moreover, the antagonistic activity of the native isolates against phytopathogenic fungi was evaluated *in vitro*. Several strains inhibited members of the genera *Fusarium*, *Sclerotinia* or *Sclerotium* and this antagonism was related to their ability to produce secondary metabolites. A phylogenetic analysis based on *rpoB* or 16S rRNA gene sequences confirmed that the isolates DGR22, MGR4 and MGR39 with high biocontrol potential belonged to the genus *Pseudomonas*. Some native strains of *Pseudomonas* were also able to synthesise indole acetic acid and to solubilise phosphate, thus possessing potential plant growth-promoting (PGPR) traits, in addition to their antifungal activity. It was possible to establish a relationship between PGPR or biocontrol activity and the phylogeny of the strains.

The study allowed the creation of a local collection of indigenous *Pseudomonas* which could be applied in agriculture to minimise the utilisation of chemical pesticides and fertilisers.

Introduction

The production of safe and affordable food for a constantly growing world population is the most important aim of agriculture [3]. However, plant pathogens, such as fungi, often limit crop yields and cause large economic losses. Therefore, farming techniques depend heavily on chemical pesticides for disease control, but it is an unsustainable strategy because of its pollution potential and the health hazards associated with pesticide use [11]. For this reason, the use of agrochemicals is declining, and new agricultural strategies have emerged for ensuring higher crop yields, while at the same time protecting human health and the environment [3,30]. Currently, certain microbial activities are being exploited in order to develop biopesticides for plant protection against several diseases. These commercial microbial pesticides include a number of well known antagonistic agents, such as *Bacillus thuringiensis*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Heliothis* NPV, *Trichoderma* spp., *Beauveria bassiana*, and *Trichgramma* [43].

Among the biocontrol rhizobacteria, those from the genus *Pseudomonas* have been broadly studied both because of their excellent combination of multiple antagonistic mechanisms against phytopathogens and in view of their well-established improvement of plant growth in the absence of pathogens [3,6]. The antagonistic ability of *Pseudomonas* spp. depends on the production of metabolites, such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), phenazine (PCA), pyoluteorin (PLT), hydrogen cyanide (HCN), and volatile compounds, as well as cell wall degrading enzymes [4,20,26]. Genes encoding DAPG, PRN, PCA, PLT and HCN have been characterised, and specific primers have been designed for detection of the *Pseudomonas* producer of these secondary metabolites [16,34,41].

The characterisation of the antifungal compounds produced by biocontrol agents and the understanding of the dynamics and composition of bacterial communities are important in order to achieve successful biological control [7]. Furthermore, a consideration of the range of environments in which a given antagonistic

Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; DAPG, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide; IAA, indole-3-acetic acid; PCA, phenazine; PGPR, plant growth-promoting rhizobacteria; PLT, pyoluteorin; PRN, pyrrolnitrin.

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agent would be used is likewise important, mainly because the climatic and/or soil conditions could influence the degree of antagonism achieved [37]. In this regard, strains isolated from the same environment where they would be employed are most effective as bioinoculants [31]. For this reason, the screening of native rhizobacteria is likely to provide potentially new biological control agents that could be employed as a strategy for plant-health protection.

Native strains of *Pseudomonas* with plant-growth-promoting rhizobacterial (PGPR) traits and the ability to suppress phytopathogenic fungi have been previously isolated from wheat in our laboratory [21]. These strains compete successfully with indigenous microflora and persist in adequate viable cell numbers in the rhizosphere of wheat [22].

In this study, a collection of strains from the *Pseudomonas* genus isolated from maize plants and bulk soil in a field of the Province of Córdoba (Argentina) was obtained. The aims of this work were: (1) to analyse the genotypic diversity of the *Pseudomonas* population through the use of repetitive-element PCR (rep-PCR) and amplified ribosomal DNA restriction analysis (ARDRA); and (2) to evaluate the antagonistic activity of these native strains against fungal phytopathogens, and characterise the mechanism responsible for their biocontrol activity. In addition, any potential relationship between the biocontrol activity and the phylogeny of the native isolates was investigated.

Materials and methods

Field sampling and bacterial isolation

Maize (*Zea mays*) plants were collected from a field of Córdoba (Argentina) at both the vegetative and reproductive stages, in December 2006 and March 2007, respectively. In December 2006, the soil contained 2.18% organic matter, 58.6 mg kg^{-1} phosphorus, 65.1 mg kg^{-1} nitrate, 14.7 mg kg^{-1} nitrate nitrogen, and it had a pH of 6.5. In March 2007, the soil contained 2.12% organic matter, 29.1 mg kg^{-1} phosphorus, 36.4 mg kg^{-1} nitrate, 8.2 mg kg^{-1} nitrate nitrogen, and it had a pH of 6.5.

Bacteria were isolated from rhizosphere, endorhizosphere and bulk soil, as described in van Overbeek et al. [44] and Berg et al. [8]. All samples were serially diluted with sterile 0.85% (w/v) NaCl solutions and plated onto King's B medium and Gould's modified S1 (mS1) medium [24], both containing cycloheximide (100 μg mL^{-1}). Plates were incubated at 30 °C. Colonies with different morphology were selected, maintained on nutrient agar (NA) at 4 °C and stored at −80 °C in 20% glycerol.

The isolates were designated in the following way: the first letter represented the sampling time (D, December; M, March), the second letter the isolation medium (K, King's B medium; G, Gould's mS1 medium), and the third letter the microenvironment (R, rhizosphere; E, endorhizosphere; S, bulk soil), with, finally, the number of the isolate.

Characterisation of isolates

Gram staining, pigment production on King's B medium, oxidase and catalase reaction, and the oxidative/fermentative metabolism of glucose were tested in all isolates. Presumptive isolates of *Pseudomonas* were confirmed by the PCR reaction with the *Pseudomonas*-specific primers Ps-for and Ps-rev [48]. The reference strains of the *Pseudomonas* genus used as positive controls were *P. fluorescens* WCS365 and *Pseudomonas putida* KT2440. In addition, the metabolic profile of the native strains was evaluated with Analytical Profile Index (API 20NE) strips (Biomerieux, 00144 Rome, Italy), according to the manufacturer's recommendations.

Repetitive-element PCR (rep-PCR) fingerprinting

Genomic fingerprints of bacterial isolates were obtained by amplification with BOX or ERIC primers [45]. The PCR reaction was carried out in a final volume of 25 μL containing 50 ng of total DNA, following the protocol of de Bruijn [15], and incubated in a thermal cycler according to the specifications of Cubero and Graham [13]. Ten microlitres of PCR products were separated on 1.6% (w/v) agarose gels. DNA-banding patterns were visualised by staining with ethidium bromide and analysed with Gelcompar II version 4.1 software (Applied Maths, Kortrijk, Belgium) through the use of the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm and Dice's coefficient for a combined gel.

Amplified ribosomal DNA restriction analysis (ARDRA)

Full-length 16S rRNA-encoding gene fragments from isolates were obtained by PCR amplification with primers rD1 and fD1 [47]. The reaction was performed in a 50 μL mixture, as described by Fischer et al. [22]. Ten microlitres of PCR products were digested overnight at 37 °C with 5 U of the restriction endonucleases *Hae*III, *Rsa*I and *Hinf*I, and at 65 °C with *Taq*I (5 U). The digests were electrophoresed in 2% (w/v) agarose gels. The resulting banding patterns were analysed through the use of Gelcompar II version 4.1 software (Applied Maths, Kortrijk, Belgium) with a band tolerance of 5%. Dendrograms were constructed by means of the UPGMA clustering algorithm and Dice's coefficient.

Sequences of 16S rDNA and rpoB genes

Some isolates were selected and sent to Macrogen Inc. (Seoul, South Korea) for the sequencing of the gene encoding 16S rRNA through the use of the primers 518F and 800R. In addition, PCRs were performed in order to amplify the *rpoB* gene through the use of the primers LAPS and LAPS27, as described by Tayeb et al. [42]. PCR products were also sequenced by Macrogen Inc. (Seoul, South Korea). Homology studies were carried out with the NCBI GenBank BLAST program [2]. The 16S rRNA and *rpoB* gene sequences of the isolates were aligned by means of Clustal W with the sequences of other *Pseudomonas* strains retrieved from the GenBank database. The construction of a neighbor-joining tree [38] and bootstrap analysis of 1000 resamplings [19] were performed.

The sequences obtained were deposited in the GenBank nucleotide-sequence database under the accession numbers HM447033, HM447034, HM447035 and JQ627866 for the 16S rRNA gene and JQ627867–JQ627870 for the *rpoB* gene.

In vitro antifungal activity

Antagonistic activity of *Pseudomonas* strains against phytopathogenic fungi (*Fusarium verticillioides* RC2000, *Fusarium solani*, *Fusarium graminearum* RC 664, *Fusarium proliferatum* RC 479, *Sclerotinia minor*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*) was tested on potato dextrose agar (PDA) and trypticase soya agar (TSA) media [29]. The incubation was performed at room temperature, and all fungi were also grown on PDA and TSA alone as a control.

Secondary metabolite production and detection of antibiotic- and hydrogen cyanide (HCN)-encoding genes

Strains with antifungal activity were tested for the production of lytic enzymes and secondary metabolites. Protease activity was determined in medium containing 3% (v/v) skim milk, β-glucanase activity was assayed in plates containing 0.1% (w/v)

lichenan (Sigma) [46], cellulase activity in medium with 2% (w/v) carboxymethylcellulose [27] and chitinase activity in medium containing 0.15% (w/v) colloidal chitin [40]. The plates were observed for degradation zones, as judged by a clearing after 1–7 days of growth at 30 °C. The production of siderophores was detected by the formation of orange halos surrounding bacterial colonies on CAS-agar plates after 48 h incubation at 30 °C [39]. In addition, the production of HCN was tested by the alkaline picrate test, as described by Bakker and Schippers [5].

The detection of genes encoding the production of 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), phenazine (PCA) and pyoluteorin (PLT) in the native *Pseudomonas* strains was carried out. For these determinations, PCR assays were performed through the use of the primers and protocols described by Raaijmakers et al. [34] and de Souza and Raaijmakers [16]. *Pseudomonas chlororaphis* strain Phz24, a producer of PRN (*prnD*$^+$) and PCA (*phz*$^+$), *Pseudomonas* sp. strain P60, a producer of DAPG (*phlD*$^+$), and *P. fluorescens* strain CHA0, a producer of PLT, DAPG and PRN (*pltC*$^+$, *phlD*$^+$, *prnD*$^+$), were used as positive controls. In addition, a PCR reaction was performed with specific primers for the detection of *hcnAB* genes (involved in the biosynthesis of the HCN synthetase critical for HCN production), as described by Svercel et al. [41]. *P. fluorescens* strain CHA0 was used as a positive control.

In all instances, 10 μL of PCR products was run on 0.8% (w/v) agarose gels.

Pyrrolnitrin extraction and detection by thin layer chromatography (TLC)

Extraction of pyrrolnitrin from strains DGR22 and MGR39 was carried out as described by Rosales et al. [36]. Briefly, the antibiotic was extracted with 5 mL acetone from the cell pellets obtained from 100 mL of culture grown in NYB broth (0.8% nutrient broth plus 0.5% yeast extract) supplemented with mannitol [18] after 5 days. Debris was removed by centrifugation at 7800 × *g* for 10 min. Subsequently, the supernatant (acetone) was removed under vacuum and the residual aqueous phase was extracted with ethyl acetate. This fraction was dried under vacuum and the residue was dissolved in methanol. The samples were spotted on silica gel 60 plates (Kieselgel 60, Merck) and developed with acetonitrile–methanol–water (1:1:1). Spots were visualised after spraying with diazotised sulphanilic acid, DSA (1% sulphanilic acid,

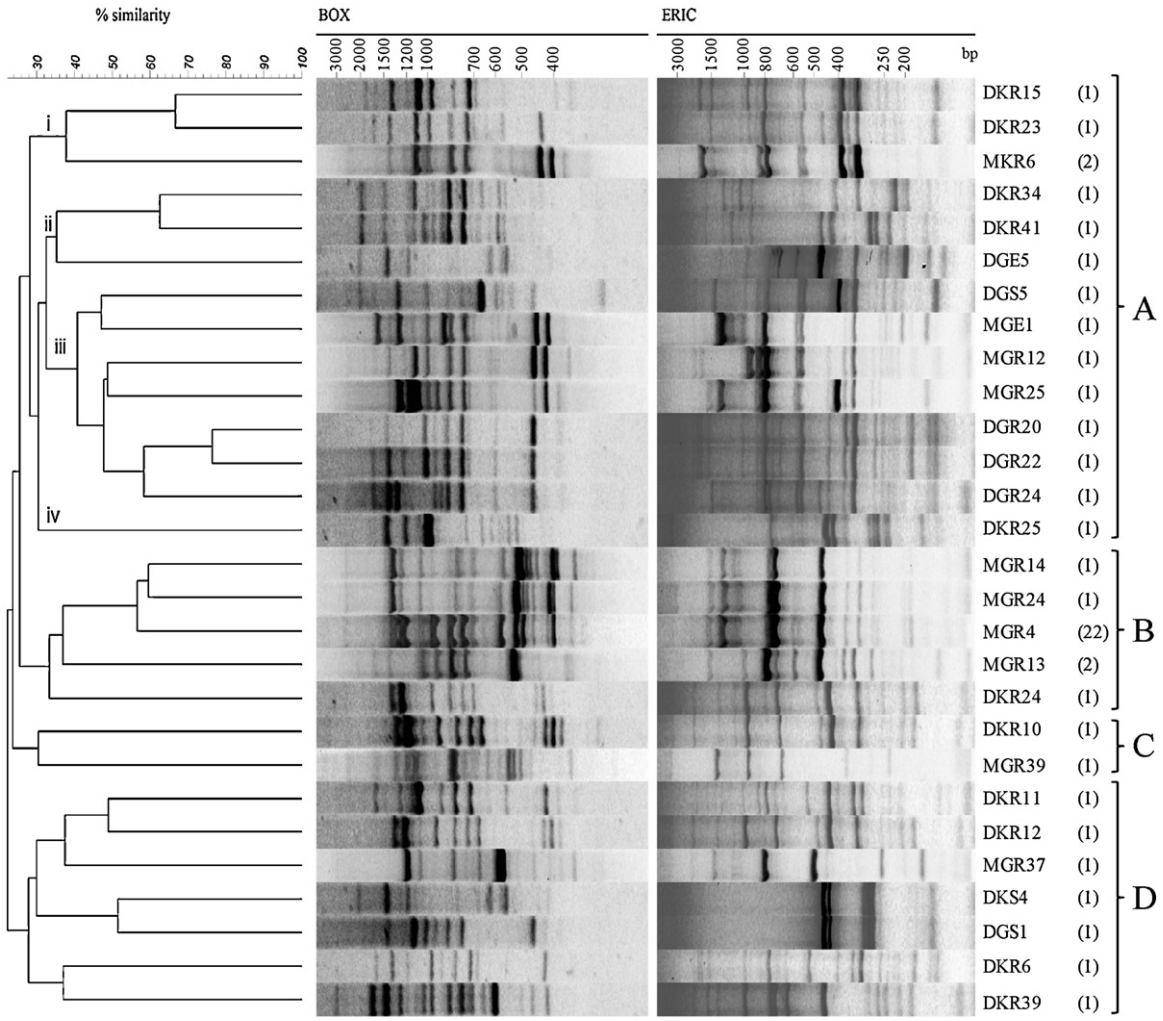

Fig. 1. Normalised band profiles from rep-PCR (BOX and ERIC-PCR) fingerprints of *Pseudomonas* isolates from endorhizosphere, rhizosphere and bulk soil of a maize field. Isolates were obtained from the vegetative (in December) and reproductive (in March) stages of plant growth. The dendrogram showing the relationships of native strains of *Pseudomonas* was built by using GelCompar version 4.1 software. A UPGMA clustering algorithm and Dice's coefficient were applied to the similarity matrix obtained based on presence or absence of bands. Groups were delineated by approximately 30% similarity. The designation of the name of the isolates is explained in the Materials and Methods section. The number of isolates showing the same fingerprint is indicated in parenthesis. The fragment sizes (bp) were calculated by the GelCompar version 4.1 software, on the basis of the molecular weight marker (O'Range Ruler 200 bp DNA Ladder), and are indicated above.

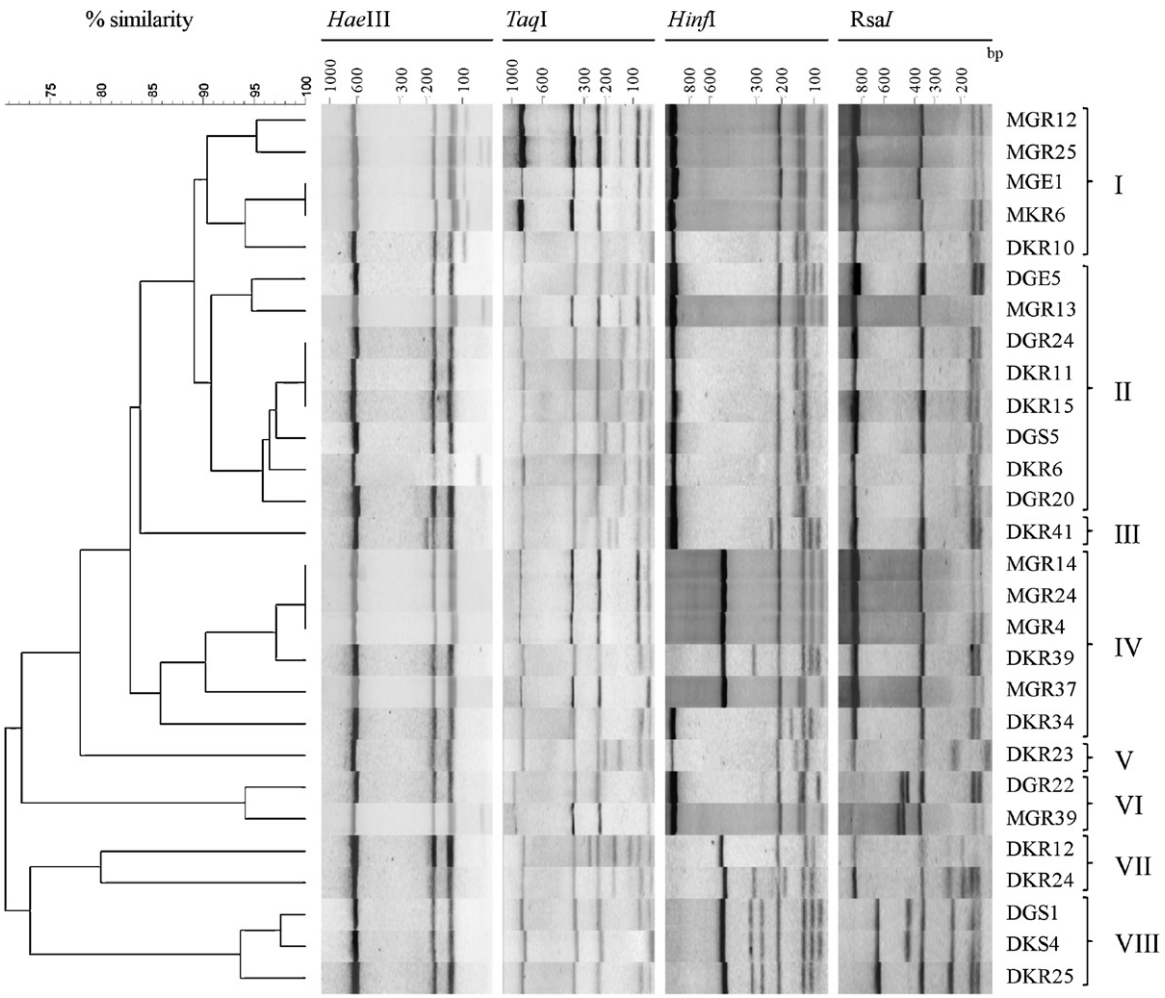

Fig. 2. Amplified ribosomal DNA restriction analysis (ARDRA) of *Pseudomonas* isolates from endorhizosphere, rhizosphere and bulk soil of a maize field. Isolates were obtained from the vegetative (in December) and reproductive (in March) stages of plant growth. The pairwise coefficient of similarity (Dice) was used for clustering with the UPGMA algorithm, using GelCompar version 4.1 software with 5% band tolerance. The designation of the name of the isolates is explained in the Materials and Methods section. The fragment sizes (bp) were calculated by the GelCompar version 4.1 software, on the basis of the molecular weight marker (O'Range Ruler 200 bp DNA Ladder), and are indicated above.

0.5% sodium nitrite and 10% potassium carbonate; in the ratio of 1:2:3). The reference strain *P. chlororaphis* Phz24 was included as a positive control.

Determination of plant growth-promoting characteristics

IAA production analysis was performed with native *Pseudomonas* spp. strains. Bacteria were cultured for 5 days in Luria-Bertani broth (without tryptophan or supplemented with 500 $\mu g\,mL^{-1}$ of tryptophan) in the dark at 30 °C. Bacterial cells were removed from the culture medium by centrifugation at 8000 × *g* for 10 min. Then, 1 mL of supernatant was mixed vigorously with 2 mL of Salkowski's reagent (4.5 g of $FeCl_3$ per litre in 10.8 M H_2SO_4) and incubated at room temperature in the dark for 30 min. The absorbance at 535 nm was measured. The concentration of IAA was determined by comparison with a standard curve. The amount of IAA produced was expressed as $\mu g\,mg^{-1}$ protein [32]. *Azospirillum brasilense* Cd and *P. putida* SF10b [21,22] were used as reference strains.

Solubilisation of phosphate was analysed in native *Pseudomonas* spp. strains by the formation of transparent halos surrounding bacterial colonies on a medium containing insoluble phosphate after 5 days incubation at 30 °C [23].

Results

Isolation and characterisation of native strains from maize plants

A total of 269 native strains were obtained from a maize field during the vegetative (December) and reproductive (March) stages of plant growth by means of selective media specific for *Pseudomonas*. The number of isolates found was higher in the rhizosphere (188) than in the bulk soil (60) or in the endorhizosphere (21). Furthermore, more isolates were recovered on King's B medium (177) than on Gould's mS1 medium (92).

Thirty-one isolates from December and 46 from March were characterised as motile Gram-negative rods, positive for catalase and oxidase, and obligate aerobes for glucose fermentation, whereas 53 isolates produced fluorescent pigment on King's B medium. A single fragment (about 1000 bp) was amplified by a PCR specific method for the *Pseudomonas* genus in 18 strains isolated in December and 34 in March.

In addition, biochemical tests were performed using the API 20NE system with 24 representative isolates obtained in December and March. All strains gave negative results for potassium nitrate, L-tryptophan, fermentation of D-glucose, urea, aesculin and adipate but were positive for L-arginine, assimilation of D-glucose,

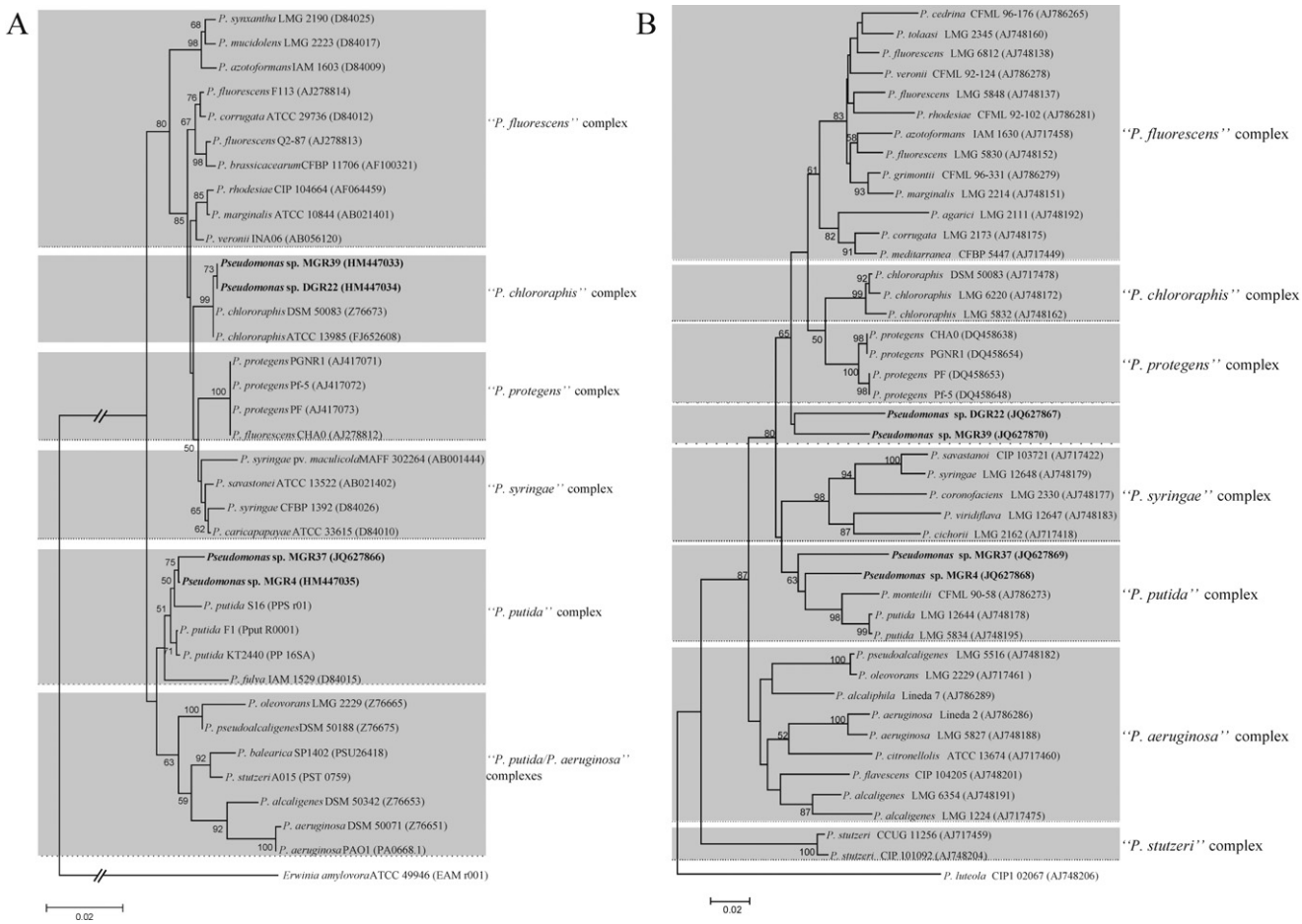

Fig. 3. Phylogenetic tree based on a comparison of the 16S rDNA (A) and *rpoB* (B) gene sequences, showing relationships between four *Pseudomonas* strains isolated from the rhizosphere of maize plants and other closely related members of the genus *Pseudomonas* retrieved from the GenBank database. The tree was constructed using the Kimura 2-parameter model and neighbor-joining methods. The bootstrap values, expressed as percentages of 1000 replicates, are given at the branching points (values lower than 50% are not shown). The scale bars show two substitution nucleotides per 100 nucleotides. The 16S rDNA and *rpoB* sequences of strains belonging to other genera were used to root the phylogenetic tree. The database accession numbers are given in parenthesis.

D-mannose, caprate, malate, citrate and oxidase. Details of the assay with the API 20NE system are given in Supplementary Table S1. These results demonstrated that 22 of the 24 isolates had a high degree of similarity to *P. fluorescens*, whereas strains MGR37 and DGS5 were identified as *P. putida* with similarity percentages of 99.7 and 99.5, respectively.

rep-PCR fingerprinting

In order to distinguish closely related strains among 51 isolates of *Pseudomonas* spp., the patterns of BOX- and ERIC-PCR were analysed (Fig. 1). Fingerprints obtained by BOX-PCR presented DNA bands ranging from 200 to 3700 bp, whereas for ERIC-PCR, the molecular weights of the amplified products were estimated at approximately 100–2100 bp. A total of 18 unique rep-PCR fingerprints were detected among the strains isolated during the vegetative-growth phase of the plants, along with 10 fingerprint profiles among the isolates obtained from the plants in the reproductive phase. A total of 22 isolates were also obtained from the reproductive phase of the plants and they exhibited an identical rep-PCR pattern. A representative strain (MGR4) within this group was selected for further characterisation.

Cluster analysis allowed the definition of four major groups (A, B, C and D) at approximately 30% similarity (Fig. 1). Cluster A was comprised of 15 strains obtained from bulk soil, rhizosphere, and endorhizosphere from both plant growth periods. This cluster could be divided into four subclusters (i, ii, iii and iv). Seven out of 15 strains were grouped in subcluster iii, whereas subcluster i consisted of strains from the rhizosphere of plants sampled in December and March. Two strains from the rhizosphere and one from the endorhizosphere of maize plants with a vegetative stage of growth were clustered within subcluster ii, while subcluster iv contained only one strain (DKR25). Most of the strains in cluster B were obtained from the reproductive stage of the plant. Contrarily, the majority of the strains in cluster D were recovered from plants with a vegetative stage of growth. Cluster C included one strain from plants sampled in December and one from March.

Amplified ribosomal DNA restriction analysis (ARDRA)

A single product of about 1.5 kb was amplified by PCR with the primers fD1 and rD1 from each of 28 *Pseudomonas* spp. isolates analysed. This product was digested with the endonucleases *Hae*III, *Taq*I, *Hinf*I and *Rsa*I. The construction of the resulting dendrogram indicated the presence of eight major groups: I, II, III, IV, V, VI, VII, and VIII (Fig. 2). Most of the strains from group I were obtained from the maize reproductive-growth phase. In contrast, in group II, the majority of strains were from the vegetative phase. Group IV comprised strains from both plant growth periods, while groups VII and VIII only had strains from the December samples. Strains DGR22 and MGR39 were clustered in group VI with a similarity of approximately 94%. Two strains (MGE1 and MKR6) from group I,

Table 1
Antagonism against different phytopathogenic fungi and secondary metabolites produced by native strains of *Pseudomonas* isolated from endorhizosphere, rhizosphere and bulk soil of a maize field.

Strain[a]	Antagonistic activity towards[b]														Secondary metabolites[c]			PCR[d]	
	Fg		*Fv*		*Fp*		*Fs*		*Ss*		*Sm*		*Sr*		S	P	C	HCN	PRN
	A	T	A	T	A	T	A	T	A	T	A	T	A	T					
DKR6	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	+	–	+	–
DKR10	+	–	–	–	–	+	–	–	–	+	–	–	–	+	–	+	–	+	–
DKR11	–	–	–	–	–	–	–	–	–	+	–	–	–	+	+	+	–	+	–
DKR12	+	+	–	+	–	+	–	–	–	+	–	–	–	+	+	+	–	+	–
DKR15	–	+	–	–	–	–	–	–	+	+	–	–	–	+	+	+	–	+	–
DKR23	–	–	–	+	–	–	–	–	–	+	–	–	–	+	–	+	–	+	–
DKR24	–	+	–	–	–	+	–	+	–	+	–	–	–	+	+	+	+	+	–
DKR25	–	–	–	–	–	–	–	+	–	–	–	–	–	+	+	–	–	–	–
DKR34	–	–	–	–	–	–	–	+	–	+	–	–	–	+	+	+	–	+	–
DKR39	–	–	–	–	–	+	–	+	–	+	–	–	–	+	+	+	–	+	–
DKR41	+	–	–	–	–	+	+	–	–	+	–	–	–	+	+	+	–	+	–
DGR20	–	–	–	–	–	–	+	–	–	+	–	–	–	+	+	+	–	+	–
DGR22	+	+	+	+	–	+	+	–	+	–	–	–	–	+	+	+	–	+	+
DGR24	–	+	–	–	–	–	+	–	–	–	–	–	–	+	+	+	–	+	–
DKS4	+	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	+	–	–
DGS1	–	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	+	–	–
DGS5	–	–	–	–	–	+	+	–	–	–	–	–	–	+	+	+	–	+	–
DGE5	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	+	–	+	–
MKR6	–	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	–	+	–
MGR 4	–	+	–	+	–	+	–	+	–	+	–	+	–	–	+	+	+	+	–
MGR12	–	–	+	+	–	–	–	–	–	+	+	+	–	+	+	+	–	+	–
MGR13	–	–	–	+	–	+	–	–	–	+	–	+	–	+	+	+	–	+	–
MGR14	–	+	–	+	–	+	–	+	–	+	–	+	–	–	+	+	–	+	–
MGR 24	–	+	–	+	–	+	–	+	–	+	–	+	–	–	+	+	–	+	–
MGR 25	–	+	–	+	–	–	–	–	–	+	+	+	–	+	+	–	+	+	–
MGR 37	–	–	–	–	–	–	–	+	–	–	–	–	–	–	+	+	+	–	–
MGR 39	+	+	+	+	+	+	–	–	–	–	–	–	–	–	+	–	+	+	+
MGE1	–	+	+	+	–	–	–	–	–	+	+	+	–	+	+	+	+	+	–

[a] The designation of the name of the isolates is explained in the Materials and Methods section.
[b] Phytopathogenic fungi evaluated were Fg, *Fusarium graminearum*; Fv, *F. verticillioides*; Fp, *F. proliferatum*; Fs, *F. solani*; Ss, *Sclerotinia sclerotiorum*; Sm, *S. minor*; Sr, *Sclerotium rolfsii*. Media used to analyse the antagonistic activity were A, potato dextrose agar; T, trypticase soya agar.
[c] Secondary metabolite production: S, siderophore; P, proteases; C, cellulase.
[d] PCR detection of genes encoding the production of HCN and PRN.
+, positive; –, negative.

three strains (DGR24, DKR11 and DKR15) from group II and three strains (MGR14, MGR24 and MGR4) from group IV showed a 100% similarity between themselves, respectively. Groups III and V were each represented by only a single strain.

Phylogenetic analysis

Strains MGR4, MGR37, MGR39 and DGR22 were selected by their antagonistic ability or by secondary metabolites production (see sections below) for phylogenetic studies. Partial sequences of the 16S rRNA gene from these strains were obtained and compared with those retrieved from the GenBank database. Sequence analysis of the four strains showed 99% identity to the 16S rRNA gene from strains belonging to the *Pseudomonas* genus. A phylogenetic tree based on 16S rDNA sequences was constructed by the neighbor-joining method. Phylogenetic analyses included sequences of representative species of all *Pseudomonas* species complexes [35]. The position of the native strains relative to members of the *Pseudomonas* genus is shown in Fig. 3A. Cluster analysis indicated a close evolutionary relationship between the native strains DGR22 and MGR39. These strains were grouped in the *P. chlororaphis* complex, while strains MGR4 and MGR37 clustered with strains belonging to the *P. putida* complex.

rpoB is a highly conserved housekeeping gene. Due to its discriminatory power, this gene has been used for phylogenetic analysis of several bacterial groups, including the strains of the *Pseudomonas* genus [42]. In this study, *rpoB* gene partial sequences (800–1100 bp) were determined in the native strains. The phylogenetic analysis of the *rpoB* gene (Fig. 3B) was similar to the results obtained with the 16S rDNA sequences in strains MGR4 and MGR37. However, although isolates DGR22 and MGR39 were closely related, they formed a new clade.

In vitro antagonistic activity towards phytopathogenic fungi

The antagonistic activity of 28 native *Pseudomonas* strains against phytopathogenic fungi was tested *in vitro* on PDA and TSA media. A variation in the antagonism of the strains of *Pseudomonas* against different fungi was observed on the different media (Table 1 and Fig. 4). Whereas the majority of the strains were able to inhibit the growth of *S. rolfsii* and *F. proliferatum* on TSA medium, none had antifungal activity against these same fungi on PDA, with the exception of MGR39 that did, in fact, inhibit the growth of *F. proliferatum* on this medium. In addition, certain strains inhibited the mycelial growth of *F. solani* on either PDA or TSA, but not on both media. *S. minor* was only antagonised by the isolates obtained in March.

Strains DGR22, MGE1 and MGR39 were considered strong antagonists since they inhibited a broad number of fungi and, in some instances, the inhibition occurred on both media. Moreover, antagonistic activity of strains MGR4, MGR14 and MGR24 was observed against all the fungi, except for *S. rolfsii* on TSA medium, whereas DKR11, DKR25, DGS1 and DGE5 inhibited the growth of only a few pathogenic fungi.

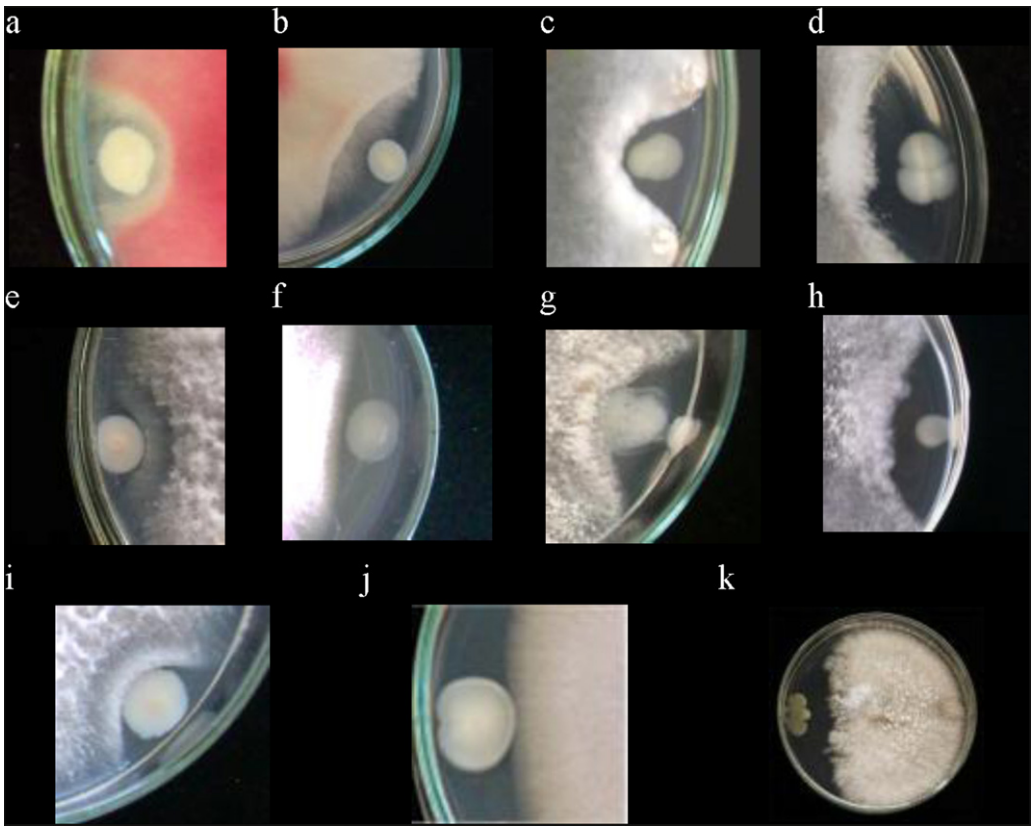

Fig. 4. Antagonistic activity of some native strains of *Pseudomonas* against phytopathogenic fungi. *Pseudomonas* sp. MGR39 vs. *Fusarium graminearum* on PDA (a) and TSA (b); *Pseudomonas* sp. DGR22 vs. *Sclerotinia sclerotiorum* on PDA (c); *Pseudomonas* sp. MGR4 vs. *S. sclerotiorum* on TSA (d); *Pseudomonas* sp. DGR22 vs. *Fusarium verticillioides* on PDA (e); *Pseudomonas* sp. MGR4 vs. *F. graminearum* on TSA (f); *Pseudomonas* sp. MGR12 vs. *Sclerotinia minor* on PDA (g); *Pseudomonas* sp. MGR25 vs. *S. minor* on TSA (h); *Pseudomonas* sp. MGR39 vs. *Fusarium proliferatum* on PDA (i); *Pseudomonas* sp. MGR24 vs. *Fusarium solani* on TSA (j); *Pseudomonas* sp. MGE1 vs. *Sclerotium rolfsii* on TSA (k).

Secondary metabolite production and PCR-based detection of genes encoding antibiotics

For a further characterisation of the antagonistic activity of native *Pseudomonas* strains, the production of secondary metabolites was investigated (Table 1). Most of the isolates were able to produce protease, while only a few could degrade cellulose. None of the strains had either chitinolytic, β-glucanase or pectinase activity. In addition, most of the strains were siderophore producers on CAS medium.

The antagonistic ability of *Pseudomonas* spp. has been associated with the production of the antibiotics DAPG, PCA, PLT and PRN. For this reason, the native strains of *Pseudomonas* spp. were screened by PCR for the presence of the biosynthetic operons encoding these four antibiotics. A single fragment of about 786 bp was amplified through the use of specific primers PRND1 and PRND2 [16] in the pyrrolnitrin-producing reference strain and in two of the native strains (DGR22 and MGR39) (Table 1). In contrast, a fragment of the expected size for DAPG, PCA, and PLT [34] was observed in the reference strains, but no such fragments were amplified from the DNA of any of the native isolates.

For detection of pyrrolnitrin production, extractions with organic solvents were carried out from the cell pellets obtained from culture of strains DGR22, MGR39 and reference strain *P. chlororaphis* Phz24. Cell extracts of two native strains yielded a metabolite on TLC plates with a relative mobility (Rf value) of 0.85. This chromatographic characteristic was similar to those of pyrrolnitrin produced by the reference strain (Supplementary Fig. S1). In addition, the antibiotic spot (both native and reference strains) turned orange with the spray reagent (DSA), indicating the presence of phenolic forms [14]. Therefore, the antibiotics produced by native strains DGR22 and MGR39 were tentatively identified as PRN. These results showed a perfect match between the results obtained in the biochemical analysis and PCR-based detection.

Moreover, HCN-producing *Pseudomonas* strains were detected through PCR and the use of the specific primer targeting the *hcnAB* genes essential for HCN biosynthesis [41]. Upon performing this assay, a single fragment of about 570 bp in length was obtained for most of the native isolates (Table 1), as well as for the reference strain (*P. fluorescens* CHA0), indicating the presence of the *hcnAB* genes in these strains. The production of HCN was assayed by the alkaline picrate test. A change in colour of the filter paper from yellow to orange confirmed the ability to produce HCN by all native strains that possessed the *hcnAB* genes (data not shown).

Identification of potential PGPR

A total of 27 native strains of *Pseudomonas* spp. were screened for their ability to produce indole-3-acetic acid (IAA)-like substances and to solubilise phosphate. All the strains, except for ten, produced more IAA when tryptophan was added to medium, indicating that they could use a tryptophan-dependent pathway for the synthesis of IAA (Table 2). Moreover, the reference strains *A. brasilense* Cd and *P. putida* SF10b produced 101.24 and 81.8 μg of IAA per mg of protein in LB medium with tryptophan, respectively. Meanwhile, the IAA production in medium without tryptophan was lower (73.57 μg of IAA per mg of protein for *A. brasilense* Cd and 46.14 μg of IAA per mg of protein for *P. putida* SF10b). It is noteworthy that native strain MGR24 synthesised more IAA than the reference strains by a tryptophan-dependent pathway (113.94 μg of IAA per mg of protein).

Table 2
Relationship between the phosphate solubilisation or IAA production and phylogeny of the native strains of *Pseudomonas* isolated from endorhizosphere, rhizosphere and bulk soil of a maize field.

ARDRA	Strains	Phosphate solubilisation	IAA production ($\mu g\,mg^{-1}$ protein)[a]	
			– Try	+ Try
I	MGR12	+	1.48	16.635
	MGR25	–	3.9	14.695
	MGE1	–	6.39	17.35
	MKR6	–	4.08	14.63
	DKR10	–	22.2	54.23
II	DGE5	–	12.76	26.4
	MGR13	–	5.16	–
	DGR24	–	7.63	23.99
	DKR11	–	32.54	26.08
	DKR15	–	17.94	13.23
	DGS5	–	25.47	17.45
	DKR6	–	8.81	14.89
	DGR20	–	11.72	25.39
III	DKR41	+	22.51	20.3
IV	MGR24	+	12.43	113.945
	MGR4	+	1.31	41.97
	DKR39	+	12.44	34.42
	MGR37	+	12.21	33.765
	DKR34	+	18.62	41.75
V	DKR23	–	21.64	17.75
VI	DGR22	–	–	–
	MGR39	–	1.18	15.785
VII	DKR12	+	22.01	57.93
	DKR24	+	20.42	42.33
VIII	DGS1	–	35.21	17
	DKS4	–	15.9	16.31
	DKR25	+	11.18	11.71

[a] – Try: LB medium without tryptophan; + Try: LB medium supplemented with 500 $\mu g\,mL^{-1}$ tryptophan.
–, no solubilisation/non-production of IAA; +, solubilisation.

In addition, 33% of the isolates from December and 45% of the strains from March were able to solubilise phosphate. There were correlations between the ability to solubilise phosphate or IAA production and the phylogeny of *Pseudomonas*. All members of ARDRA groups IV and VII were phosphate solubilisers and the best IAA producers (Table 2).

Discussion

In this study, bacteria were isolated on King's B and Gould's mS1 media from bulk soil, rhizosphere and endorhizosphere of maize crops from the central region of Argentina. A lower number of isolates was obtained on Gould's mS1 than on King's B medium mainly because Gould's mS1 medium is more selective, less susceptible to overgrowth by non-pseudomonads and more reproducible than King's B medium [25,28]. Furthermore, most of the strains were obtained from the rhizosphere samples. Similar results have been observed previously with other strains of *Pseudomonas*. For example, Berg et al. [9] isolated more *Pseudomonas* strains from the rhizosphere of strawberry or oilseed rape than from the other microenvironments in these plants.

Genomic analysis based on the rep-PCR method indicated a lower level of diversity for isolates obtained from plants in the reproductive stage of growth than for isolates obtained from plants in the vegetative stage of growth. Moreover, all the strains from December presented different fingerprints, whereas 22 isolates from March had an identical genotype, indicating that the same strain was recovered several times. This strain might therefore be better adapted to, or be more competitive in, the root environment of the reproductive plants from March. These results were similar to those observed in populations of *Burkholderia cepacia* and *Pseudomonas*, where a greater diversity was found in young plants than in mature ones [12,17].

On the basis of rep-PCR and ARDRA analyses, it was observed that strains did not cluster according to the microenvironment or the phenological stage of maize plants. In addition, a low relationship was found between the groups defined by rep-PCR and those obtained by ARDRA. These results agree with Alippi et al. [1] who found no correlation between fingerprint patterns obtained by rep-PCR and ARDRA in populations of phytopathogenic *Pseudomonas* isolated from tomato and pepper. This lack of correlation is expected since ARDRA generally provides a resolution at the species level, whereas the fingerprinting techniques, such as rep-PCR, allow an identification of different subspecies of bacteria [33].

The rhizosphere is an important reservoir of *Pseudomonas* strains that exhibit antagonistic traits [9]. For this reason, the antagonistic potential of the native isolates was evaluated against a broad number of phytopathogenic fungi on TSA and PDA media. Variation in the antagonism of the native strains against fungi was observed on different solid media. These results are in agreement with McSpadden-Gardener et al. [29], who found that medium composition influenced the pathogen-inhibition activities expressed by different *phlD*$^+$ genotypes of *Pseudomonas*. Therefore, the biocontrol activities of these pseudomonads could probably vary among different crops [29].

In addition, the secondary metabolites produced by the native isolates, which could be related to antagonistic activity, were also analysed. It is important to remark that strains inhibiting a broad number of phytopathogenic fungi were able to produce more than one secondary metabolite (proteases, celluloses, siderophore and HCN). Moreover, both genetic and biochemical evidence were obtained for pyrrolnitrin production by strains DGR22 and MGR39. Similarly, Costa et al. [12] found that the majority of the *Pseudomonas* spp. strains displaying antifungal activity against *Verticillium dahliae* and *Rhizoctonia solani* were siderophore and protease producers, but only one of the thirteen strains was positive for the detection of the PRN antibiotic-encoding gene by PCR and two for the detection of DAPG encoding genes. Moreover, Garbeva et al. [25] observed, in a collection of 210 *Pseudomonas* isolated from soil, that 18% of the strains were *prnD*$^+$, while 12% of the isolates showed a positive signal for PCA and only 1% of the strains were *phlD*$^+$.

An attempt was made to establish a relationship between biocontrol activity and the phylogeny of the native isolates. In general, it was observed that most of the strains clustering in ARDRA group IV had the ability to produce siderophores and lytic enzymes and were therefore good antagonists. Accordingly, the most effective isolates for inhibiting fungi were strains DGR22 and MGR39 (both produced PRN), and they clustered in group VI with high similarity values. Moreover, the phylogenetic analysis based on 16S rDNA and *rpoB* sequences grouped both strains in the same clade. In contrast, group II contained strains having either limited antagonistic effect or none at all.

Recently, Ramette et al. [35] taxonomically characterised *Pseudomonas* strains producing the antibiotics DAPG (Phl$^+$) and pyoluteorin (Plt$^+$). Based on 16S rRNA, *rpoB*, *rpoD* or *gyrB* phylogenies, all Phl$^+$ Plt$^+$ *Pseudomonas* strains were clustered into a single and novel clade, and these authors proposed the species name *Pseudomonas protegens* sp. nov. for them. In contrast, all Phl$^+$ Plt$^-$ strains clustered within the "*P. fluorescens*" cluster [35].

In addition, a relationship was also observed between the ability to solubilise phosphate or IAA production and the phylogeny of the native strains. These results are in agreement with Browne et al. [10] who demonstrated that *Pseudomonas* strains with the ability to solubilise phosphate were linked to a single phylogenetic lineage within the *P. fluorescens* complex.

The findings of this study should aid in furthering our information on the genotypic characteristics and biocontrol potential of *Pseudomonas* strains isolated from the central region of Argentina. In addition, the isolates obtained will serve to enrich the collection of local strains that can be employed for the protection of crops through strategies that will at the same time reduce the need to employ fumigants, pesticides, and other chemicals that are harmful to the environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2012.04.005.

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